Molecular Epizootiology: Assessment of Exposure to Genotoxic Compounds in Teleosts

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The recent development of techniques to measure levels of carcinogens covalently bound to DNA provides the opportunity to use DNA adducts as molecular dosimeters of exposure to environmental carcinogens and mutagens. This is especially important because epizootiologic studies have shown a positive association between environmental carcinogens, such as polycyclic aromatic hydrocarbons, and increased prevalence of neoplasms and related lesions, primarily in liver, of benthic fish species from a wide range of urban and industrialized areas. In studies with wild fish and mammalian species the ^{32}P-postlabeling assay, as developed for aromatic compounds, has been used most extensively because of its high sensitivity and ability to detect structurally uncharacterized adducts. The results to date of field and laboratory studies show that hepatic DNA adducts detected in fish are associated with increased exposure to environmental polycyclic aromatic compounds in the preponderance of species examined, whereas in the limited studies with wild mammals, such a relationship is equivocal at present. The findings with fish suggest that DNA adducts, as measured by ^{32}P-postlabeling, have the potential to be effective molecular dosimeters of exposure to environmental carcinogenic aromatic compounds and thereby may lead to an improved understanding of the etiology of neoplasia in wild teleosts. — Environ Health Perspect 102(Suppl 12):19–23 (1994)

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Introduction

Studies with a wide range of fish species have shown that neoplasms are observed in discrete geographic areas near industrial activities and large urban centers (*1*). Hepatic neoplasms are the most commonly detected lesion; however, epidermal neoplasms and other extrahepatic neoplasms are observed. Moreover, in Atlantic tomcod (*Microgadus tomcod* Walbaum) from the Hudson River, New York, and winter flounder (*Pseudopleuronectes americanus*) from Boston Harbor, Massachusetts, activated Ki-*ras* oncogenes are present in hepatic neoplasms (*2,3*). In several studies, the prevalences of hepatic neoplasms show a positive statistical association with the concentrations of polycyclic aromatic hydrocarbons (PAHs) in sediment (*4–6*). Analyses of the sediments have revealed the presence of a broad range of PAHs, including several carcinogens such as benzo[*a*]pyrene and benzo[*b*]fluoranthene (*7*). Furthermore, laboratory studies (*7–9*) have shown that fish biotransform carcinogenic PAHs to DNA-reactive diol-epoxides, which are suspected of being the ultimate carcinogenic forms of PAHs having an angular benzene ring (*10*). In addition, it has been shown that exposure of fish to carcinogenic PAHs can induce hepatic neoplasia (*9,11–13*). Collectively, these laboratory findings support the positive statistical associations observed between neoplasia and environmental PAH exposure and suggest a causal relationship. Recent data from multiyear studies of marine fish have shown that, in some species, an increased risk of hepatic lesions, including neoplasms, was also associated with sediment and tissue concentrations of chlorinated pesticides, such as DDTs (*14*), and in some cases with PCBs (*5*).

Chemical carcinogenesis is a multistep process involving both genetic and epigenetic aberrations, and environmental contaminants have been shown to elicit effects at several steps (*15*). Currently, epizootiologic studies are limited in their ability to establish causality to anthropogenic chemicals in the etiology of neoplasia in fish. A limiting factor has been the lack of data on internal dose of chemical carcinogens, such as PAHs, and particularly their interaction with DNA. Covalent modification of DNA by a carcinogen is believed to be a necessary initial step in chemical carcinogenesis. Formation of carcinogen–DNA adducts involves exposure; absorption; biotransformation to specific metabolites; and lack of immediate repair of the adduct and, thus, measurements of the level of adducts reflects an integration of these processes.

Recent advances in analytic methods and molecular biology are providing the techniques to measure the interaction of carcinogens or their metabolites with DNA (*16*). The ability to measure the levels of carcinogen–DNA adducts allows the use of DNA adducts as molecular dosimeters of exposure to carcinogens, and thereby provides a better assessment of an organism's exposure to a carcinogen than measuring environmental levels of the carcinogen (*17*). Furthermore, it is believed that the use of molecular dosimetry will increase our understanding of the mechanism of chemical carcinogenesis and thereby lead to improved assessment of risk (*15,17*).

Analytic Methodology

Several analytic methodologies for measuring carcinogen–DNA adducts are being developed and applied to studies with humans (*16*). Techniques receiving considerable attention are immunoassays, electrochemical detection, fluorescence spectroscopy, mass spectroscopy and ^{32}P-postlabeling. In studies with fish and

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other wild species, the ^{32}P-postlabeling assay, as developed for measuring hydrophobic aromatic adducts such as those arising from PAHs, has received the most attention (*7,18,19*), although HPLC with fluorescence detection has also been used to estimate levels of benzo[*a*]pyrene–DNA adducts in beluga whales and fish (*20*). The primary reasons for use of the ^{32}P-postlabeling assay is its high sensitivity (1 adduct in 10^9–10^{10} nucleotides), requirement for low microgram amounts of DNA, and the ability to detect carcinogen DNA adducts of unknown structure. The latter attribute is a particular strength of the ^{32}P-postlabeling assay, because in many cases exposure is to a complex mixture of genotoxic compounds that is often not well-characterized. Conversely, given this lack of specificity, the identification of individual adducts is difficult without independent confirmation of structure.

The basis of the ^{32}P-postlabeling technique is incorporation of ^{32}P into deoxynucleotides after the DNA has been isolated, and chromatography to resolve the adducts (*21*). Briefly, the method involves isolation of the DNA, enzymatic digestion to nucleoside 3′-monophosphates, adduct enrichment by extraction of adducts into *n*-butanol or selective hydrolysis of unmodified nucleotides to nucleosides by nuclease P1, ^{32}P-phosphorylation to yield 3′,5′(^{32}P)-bisphosphates, and finally chromatography on anion-exchange thin-layer plates. The levels of adducts are then determined by quantitating the level of ^{32}P. The recent application of storage phosphor-imaging techniques increases the sensitivity, improves estimation of background, and allows generation of permanent high-resolution maps of chromatograms that are amenable to image analysis and retrospective analyses (*22*).

The ^{32}P-postlabeling method is currently only semiquantitative in most cases. The efficacy of the labeling steps, which are enzyme mediated, and the chromatographic behavior of individual adducts cannot be assessed except for a very few compounds because of the lack of reliable carcinogen–DNA adduct standards. Recently, however, interlaboratory comparison exercises (*23*), showed that agreement between laboratories was reasonable, considering the above current limitations in assessing accuracy and precision, and that the use of carcinogen–DNA adduct standards could increase markedly the accuracy of measuring specific adducts.

Recent studies with mammals suggest that coupling ^{32}P-postlabeling with HPLC improves the ability to characterize individual adducts (*24*). In addition, using immunoaffinity chromatography and HPLC coupled with either UV detection (*25*) or ^{32}P-postlabeling (*26*) has provided greater specificity in identification of individual carcinogen–DNA adducts. However, given the considerable time required for the development and characterization of antibodies to specific carcinogen–DNA adducts, additional information on the identity of major DNA adducts detected in organisms exposed to complex mixtures of environmental contaminants is required before an objective selection of target carcinogens for development of immunologic methods can be made.

DNA Adducts in Fish

The ^{32}P-postlabeling assay has been used to measure DNA adducts in wild fish from a broad range of geographically distinct areas. In most fish species, the levels of hepatic DNA adducts are greater in fish from urban and industrialized areas than in fish from minimally contaminated sites (*7,18,19,27,28*). For example, in a recent study (*29*) of oyster toadfish (*Opsanis tau*) from a well-defined gradient of creosote contamination in the Elizabeth River, Virginia, the levels of hepatic DNA adducts increased both with increasing concentrations of sediment PAHs and levels of fluorescent aromatic compounds in bile, a semiquantitative measure of recent exposure to polycyclic aromatic compounds, primarily PAHs (*30*). These findings are corroborated by laboratory studies with an organic-solvent extract of sediment from urban/industrialized area that show a dose responsive increase in hepatic DNA adducts in winter flounder (Figure 1*A*).

Representative chromatograms of hepatic DNA digests from three fish species from contaminated and reference sites are shown in Figure 2. A characteristic diagonal radioactive zone is evident in all species from the contaminated sites, and is indicative of exposure to complex mixtures of compounds. This zone, which exhibits few distinct spots, consists of multiple overlapping "spots" that can be resolved into distinct radioactive spots using different chromatographic solvent systems, as shown in Figure 2*C*. The complexity of the adduct profiles in fish from contaminated areas highlights the difficulty in identifying individual adducts. However, in light of the complex profile of polycyclic aromatic compounds observed in sediment from most contaminated sites, the ^{32}P-postlabeling assay is appropriate for the initial assessment of exposure to myriad environmental genotoxic compounds.

A recent laboratory study (*31*) has shown that a substantial portion (32 to 36% of maximum adduct levels) of hepatic DNA adducts in English sole exposed to the carcinogens benzo[*a*]pyrene and dibenzo[*c,g*]carbazole were persistent for greater than 84 days following a single exposure. These results were recently corroborated in winter flounder exposed to an organic-solvent extract of a PAH-contaminated sediment extract, which also showed the persistence of hepatic DNA adducts (Figure 1*B*). In addition, in English sole captured live from the contaminated Duwamish Waterway and held in the labo-

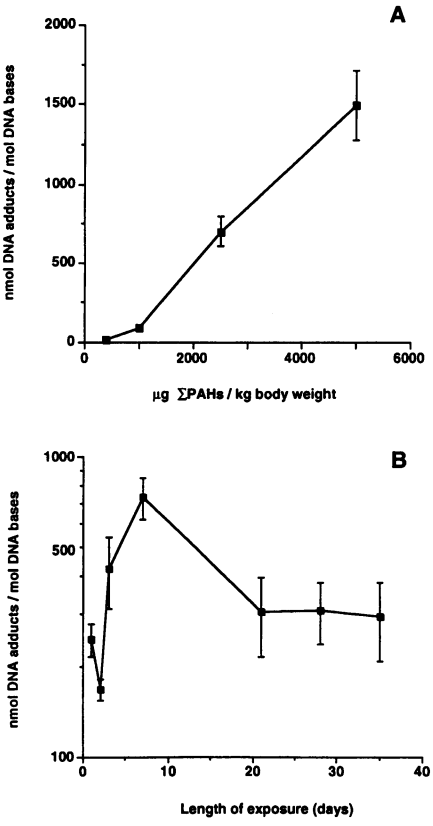

Figure 1. Levels (mean ± SE) of total hepatic DNA adducts in winter flounder exposed (intramuscularly) to an organic-solvent extract of a PAH-contaminated sediment from Puget Sound, Washington. (*A*) Relationship between DNA-adduct levels and dosage, expressed as the sum of PAHs analyzed in the extract, for fish sampled 3 days after exposure, and (*B*) time-course of DNA adducts in fish following a single dose (2.5 mg ΣPAHs/kg bw) of the sediment extract.

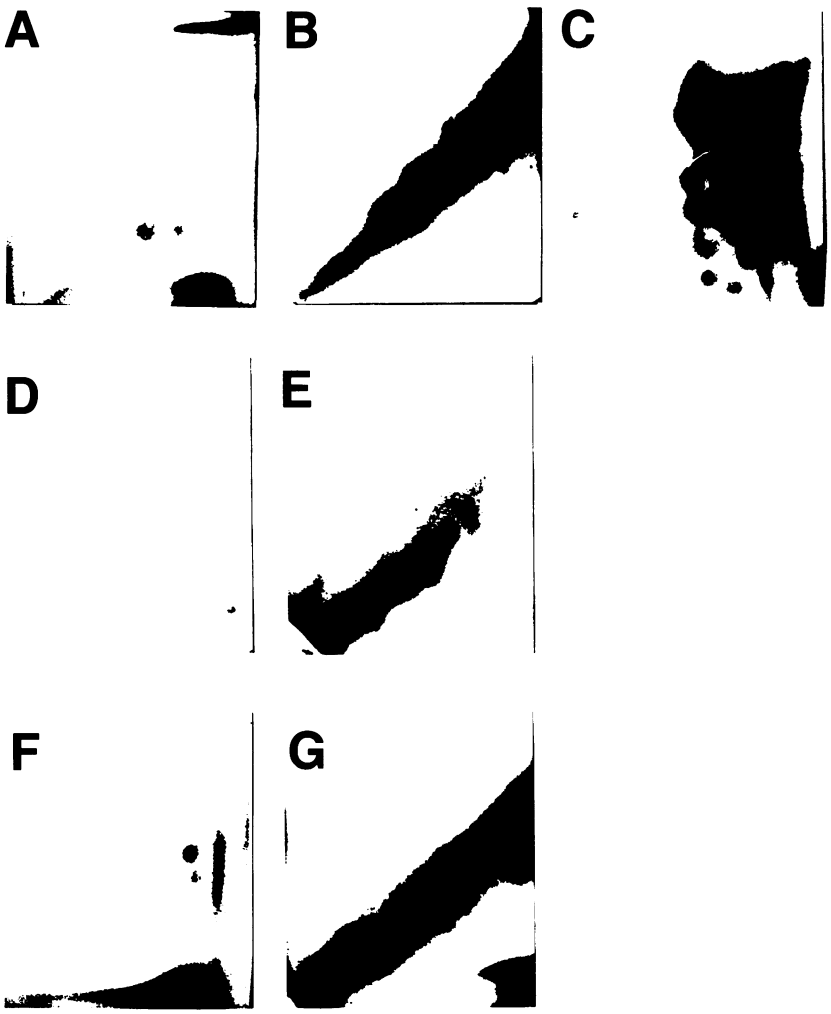

Figure 2. Representative autoradiographs of thin-layer chromatograms of hepatic DNA digests from black croaker (*A – C*), Atlantic tomcod (*D, E*), and gulf killifish (*Fundulus grandis*) (*F, G*) from minimally contaminated sites (outer Mission Bay, California (*A*); Margaree River, New Brunswick, Canada (*D*); and Little Manatee River, Tampa Bay, Florida (*F*) and contaminated sites (San Diego Harbor, California (*B,C*); Hudson River, New York, New York (*E*); North Hillsborough Bay, Tampa Bay, Florida (*G*). The chromatograms were developed in the same solvent systems except for (*C*), which was developed in D4 (left to right) using isopropanol:ammonia (1:1). The origin is located at the bottom left-hand corner. All DNA samples were analyzed by the nuclease P1 version of the ^{32}P-postlabeling assay, as described in Stein et al. (*31*), and adducts were detected by storage phosphor imaging (*22*).

ratory, greater than 50% of the DNA adduct levels present at capture were present at 60 days after capture (TK Collier, unpublished data). These findings show that a substantial proportion of DNA adducts detected in teleost liver by ^{32}P-postlabeling are not readily lost from hepatic DNA. This persistence of DNA adducts indicates that adducts would accumulate to relatively high levels in fish chronically exposed to genotoxic compounds. Such knowledge of the toxicokinetics of DNA adducts in fish is information necessary for interpreting data from epizootiologic studies.

DNA Adducts in Aquatic Mammals

Studies with mammals (*32*) have shown that apparent indigenous adducts, termed I-spots, are observed in animals not treated with carcinogens. We have consistently found, however, that the levels of adducts in fish from minimally contaminated areas are at or below the limit of detection [(*18*); Wirgin et al., unpublished data]. A study of aquatic species from the former Yugoslavia suggests, however, that indigenous adducts may be present in a fish species, and that elevated levels coincided with gonadal development (*33*). The lack of appreciable levels of suspected indigenous adducts in most fish species examined, to date, suggests a difference between teleosts and mammals in their formation. This is consistent with studies (*34,35*) with feral muskrats (*Ondatra zibethicus*) and stranded beluga whales (*Delphinapterus leucas*) in which no apparent relationship was demonstrated between the level of modification of DNA and the level of environmental contamination. In addition, our recent studies with marine mammals have shown that in some cases the majority of adducts present in liver may not be of xenobiotic origin (Reichert, unpublished data). Thus, the origin of DNA adducts in marine mammals using ^{32}P-postlabeling would appear to be equivocal at present. Overall, the results from field and laboratory studies to date show that hepatic DNA adducts detected by ^{32}P-postlabeling in fish are indicative of exposure to environmental genotoxic compounds, and that, if present, putative indigenous adducts in liver are most often at very low levels and therefore do not generally present a confounding problem in interpreting DNA adduct data. In contrast, the utility of the ^{32}P-postlabeling assay in studies with feral mammals requires further evaluation.

DNA Adducts as Molecular Dosimeters

The application of hepatic DNA adducts as molecular dosimeters in epizootiologic studies is in its infancy. In 1992, annual collection of liver tissue from several fish species for both measurement of DNA adduct levels and histopathologic examination was initiated as part of the National Benthic Surveillance Project, a component of NOAA's National Status and Trends Program. The results from these samples, together with data on hepatic concentrations of PCBs and chlorinated pesticides, such as DDT and its metabolites, will be used to assess further the relationships among exposure to environmental contaminants and prevalences of neoplastic and related hepatic lesions in fish.

Preliminary results for two species sampled on the U.S. west coast indicate that hepatic DNA adducts will be useful in assessing exposure to environmental carcinogens and their association with hepatic neoplasia. The results in Figure 3 show that in San Diego Harbor, California, a contaminated site (*36*), the level of hepatic DNA adducts and prevalences of three categories of hepatic lesions in black croaker (*Cheilotrema saturnum*) were both elevated

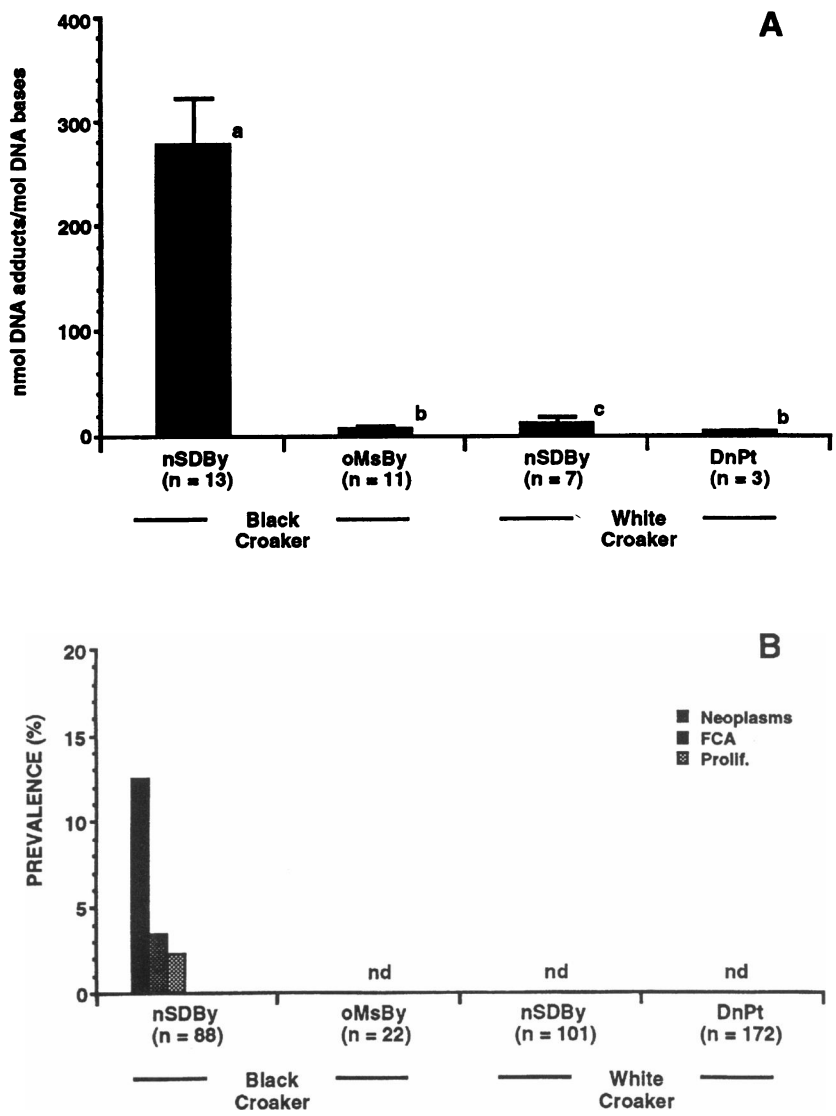

Figure 3. Levels of hepatic DNA adducts (*A*) and prevalences of hepatic lesions (*B*) in black croaker and white croaker from the contaminated San Diego Harbor, California, and from the minimally contaminated sites, outer Mission Bay and Dana Point, California. In chart *A*, common letters indicate means that were not significantly different ($p > 0.05$). The fish analyzed for adducts were sampled in 1991 and 1992, and the data on lesion prevalences are for fish sampled from 1984 to 1988 (*5*). Histopathological analyses of fish sampled in 1992 are currently in progress. FCA, putatively preneoplastic hepatic foci of cellular alteration; Prolif., nonneoplastic, nonpreneoplastic proliferative lesions.

compared to the values for white croaker (*Genyonemus lineatus*), whereas in both species the level of adducts and prevalences of these lesions in fish from a less contaminated site were low or undetected. These results suggest that the difference in lesion prevalence may, in part, be related to differences in exposure to environmental carcinogens. In addition, these results suggest that the use of DNA adducts as molecular dosimeters may significantly improve our understanding of the role of environmental carcinogens in the etiology of neoplasia in wild fish species.

Conclusion

In conclusion, the studies using the ^{32}P-postlabeling assay demonstrate the potential of hepatic DNA adducts as molecular dosimeters of exposure of teleosts to environmental genotoxic compounds. Our understanding of the etiology of hepatic neoplasia in wild fish species and of the basis for species differences in susceptibility to chemical carcinogenesis should be increased by having a more accurate assessment of exposure to genotoxic compounds than is provided by measuring environmental (e.g., sediment) levels of anthropogenic carcinogens and mutagens. However, this is not to suggest that the current state of development of methodologies for measuring DNA adducts for use in ecotoxicologic studies is sufficient. Currently, no individual DNA adducts have been unequivocally identified in a wild teleost. The identification of specific DNA adducts and their relation to subsequent genetic alterations (e.g., mutations in protooncogenes) and to neoplasia will be needed to establish more clearly causal relationships in wild species. Furthermore, the application of DNA adducts as molecular dosimeters of exposure to genotoxic compounds is not limited to molecular epizootiological studies of chemical carcinogenesis. The ability to link contaminant-induced genetic alterations to physiological effects leading to altered health and survival (*37*) will also require information on the level of interaction of genotoxic compounds with DNA. Moreover, it is anticipated that the use of DNA adducts and other biologic markers of genotoxicity will assist in the identification of causal relationships between exposure to genotoxic contaminants and increased risk of effects on individuals and populations that may lead to decreased ecologic integrity (*38*). Implicit in this is the anticipation that the use of biomarkers will increase our understanding of mechanisms by which carcinogens and mutagens exert deleterious biologic effects.

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